Supplemental Methods

Hematological and iron studies

Blood samples were obtained by retro-orbital puncture under anesthesia. CBCs were measured on an Advia 120 analyzer (H-System; Bayer Corp.). The iron content of the liver and spleen was determined by non-heme iron analysis[1,2]. Serum iron and Tf saturation levels were measured as previously described[2] using the Iron/TIBC Reagent Set (Pointe Scientific Inc.). The studies on the role of tissue iron in the recovery from anemia after BA injection were carried out using a defined diet containing 2.5-ppm iron (iron-deficient; Harlan Laboratories Inc.) or a diet supplemented with 2% carbonyl iron (iron-enriched). Control mice were fed a commercial rodent diet containing 200-ppm iron (regular), which is more than necessary for normal physiological requirements.

Quantitative PCR

RNA from liver samples was extracted using the TRIzol reagent (Sigma) according to the manufacturer's instructions, and then quantified and retrotranscribed using the SuperScript III kit (Invitrogen) according to the instructions provided. Quantitative PCRs for mouse hepcidin and for the endogenous controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, were performed as previously described[2].

Analysis of BFU-E and CFU-E

Hematopoietic progenitors (BFU-E and CFU-E) in the BM were counted by colony-forming cell assay, at 4, 14, and 24 hours after BA injection. Briefly, $5x10^4$ cells from the BM of PBS- and BA-treated WT, *IL-6*-KO, and *Hamp*-KO mice were plated in MethoCult™ M3334 media (StemCell Technologies) according to the manufacturer instructions. CFU-E and mature BFU-E were counted at day 2 and day 6 of culture, respectively. CFU-E were also determined by flow-cytometry using a FACSCalibur instrument (BD) and staining $3x10^6$ BM cells from PBS- and BA-treated WT, *IL-6*-KO, and *Hamp*-KO mice. Cell staining was performed with FITC-conjugated CD71, PE-conjugated CD41, PE-Cy7-conjugated FcGR, Alexa Fluor 467-conjugated CD105, Alexa Fluor 700-conjugated Ter119, APC-Cy7-conjugated Sca1, Brilliant Violet 421-conjugated CD150, and Brilliant Violet 605-conjugated cKit antibodies, with the addition of PerCP-Cy5.5-conjugated Lineage antibody cocktail[3] (BD Pharmingen). Results were analyzed with FlowJo software (Tree Star, Inc.).

Flow cytometry analyses of apoptosis and ROS

To determine apoptosis we stained BM and spleen cells with a FITC-Lactadherin antibody[4] (Haematologic Technologies, Inc.) together with 7-AAD (BD Pharmingen) according to the instructions provided by the manufacturers. Lactadherin is a glycoprotein originated from macrophages that binds phosphatidylserine (PS) on the outer cell membrane of apoptotic cells. Reactive oxygen species (ROS) were detected with the indicator 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluoresceindiacetate, acetyl ester (CM-H2DCFDA, Life Technologies), which permeates into the cells and is oxidized in the presence of ROS. Cells were sorted using a FACSCalibur instrument (BD) and the results analyzed with FlowJo software (Tree Star, Inc.).

Analysis of the effect of stress erythropoiesis on BA-induced anemia

To study the effect of stress erythropoiesis on anemia induced by BA, WT mice were bled for two consecutive days (400μL of blood/25 g mouse), injected with $5x10^8$ particles/mouse of BA at day 4 and successively sacrificed for analysis 3 days after BA injection. BM and spleen cells were harvested and analyzed for erythroid progenitors cell population using FITC-labeled anti-mouse CD71, PE-conjugated anti-mouse CD44, and APC-conjugated anti-mouse Ter119 antibodies (BD Pharmingen) on a FACSCalibur instrument (BD) as previously described. FlowJo software (Tree Star, Inc.) was used for analysis.

Analysis of red blood cell lifespan

To determine the lifespan of RBC from BA-treated WT, *IL-6*-KO, and *Hamp*-KO mice we used the EZ-link-sulfo-NHS-biotin labeling kit (Thermo Scientific) that binds to the surface of erythrocytes, which provides a way to monitor the ratio between the biotinylated and the new non-biotinylated circulating RBCs. A single dose of 1 mg of sulfo-NHS-biotin was injected into mice intravenously. After 24 hours, a drop of blood was collected from the tail, incubated with FITC-conjugated streptavidin, and then analyzed by flow cytometry. FACS analyses were performed at 4, 7, 11, and 14 days after injection of BA to measure the progressive clearance of the biotin-labeled RBCs from circulation.

Supplemental References

1. Torrance JD, Bothwell TH. Tissue iron stores. Iron Methods in Hematology. Vol. 1. New York; 1980:90-115.
2. Gardenghi S, Marongiu MF, Ramos P, et al. Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. *Blood*. 2007;109(11):5027-5035.
3. Pronk CJ, Rossi DJ, Mansson R, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell stem cell*. 2007;1(4):428-442.
4. Dasgupta SK, Guchhait P, Thiagarajan P. Lactadherin binding and phosphatidylserine expression on cell surface-comparison with annexin A5. *Translational research : the journal of laboratory and clinical medicine*. 2006;148(1):19-25.

Supplemental Figures Legends

Figure S1. Erythropoiesis is severely impaired in BA-treated mice. FACS analysis of BM and spleen of WT, *IL-6*-KO, and *Hamp*-KO mice, 3 days after BA injection. CD71 (Tf receptor), Ter119 (erythroid-specific), and CD44 (adhesion molecule that exhibits a progressive decrease from pro-erythroblast to reticulocyte) co-staining was used. $CD71^+/Ter119^+$ and $CD71^-/Ter119^+$, characterizing erythroid precursor and mature red cells respectively, were selected and plotted as CD44 vs. FSC, to show the different stages of erythroid maturation. Maturation stages occurs as indicated by the arrow, and include pro-erythroblast (I), basophilic (II), polychromatic (III), orthochromatic - reticulocytes (IV), and mature erythrocytes (V). The percentage of cells constituting each population is shown. Three mice per each group were analyzed; one representative mouse per each group is shown.

Figure S2. Erythropoiesis is severely impaired in BA-treated mice. Total number of progenitor erythroid cells (population I, II, III and IV) and RBCs (populations V) of mice shown in Figure 4. WT, *IL-6*-KO, and *Hamp*-KO mice were analyzed (A) 3 days and (B) 1 week after BA injection. Graphs indicate the average number and standard deviations of three mice per group. Error bars represent SD. *P* values were calculated using unpaired, 2-tailed Student's *t* test. **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure S3. Increased apoptosis and ROS affect erythroid progenitors of mice treated with BA. WT, *IL-6*-KO, and *Hamp*-KO mice (analyzed in Figure 4 for erythroid cell precursors), were also used to measure (A) apoptosis and (B) ROS in their spleen by flow cytometry, using markers for lactadherin and CM-H2DCFDA respectively. Three mice per each group were analyzed (BA, in blue vs. PBS controls, in red). One representative mouse per each group is shown. All mice showed an increase in apoptosis and ROS production following treatment with BA, especially in their orthochromatic cells of the spleen (population IV of Figure 4).

Figure S4. Early BFU-E and CFU-E erythroid progenitors were decreased in mice injected with BA. The number of BM hematopoietic BFU-E and CFU-E progenitor cells was determined by colony-forming cell assay and by flow cytometry (CFU-E only – also see Supplemental Figure 5) at 4, 14, and 24 hours after BA injection in WT, *IL-6*-KO, and *Hamp*-KO mice. Both BFU-E and CFU-E were reduced 14 hours after BA in WT mice, and 24 hours after BA in *IL-6*-KO, and *Hamp*-KO mice. (A) Number of BFU-E measured by colony-forming cell assay. Graphs indicate the average number and standard deviations of two mice per group, plated in triplicate. (B) Number of CFU-E measured by flow cytometry. Graphs indicate the average number and standard deviations of three mice per group. Error bars represent SD. *P* values were calculated using unpaired, 2-tailed Student's *t* test. **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure S5. Analysis of CFU-E by flow cytometry. This procedure was based on the protocol described by Pronk et al.[3] and allowed discrimination and quantification of early CFU-E progenitor cells in mouse BM. Gating-steps were performed as follows: after the exclusion of debris and multiplets (i and ii), we selected the cKit+, Lin-, Sca- (KLS-) myeloerythroid population (iii and iv). Using the simultaneous assessment of cell-surface expression of CD150, CD41, FcGR, and CD105, we respectively selected $CD150^{-}/CD41^{-}$ cells (MPK– population) (v), $FcGR^{-}/CD150^{-}$ cells (GMP– population) (vi), and $CD105^{+}/CD150^{-}$ cells (vii). From this last population, we were able to identify $CD71^{+}/Ter119^{-}$ CFU-E progenitor cells (viii), which were plotted as FSC vs. SSC (ix) and quantified as number of event. Three mice per each genotype (WT, *IL-6*-KO, and *Hamp*-KO) were analyzed. The figure shows an example of analysis performed in (A) PBS-treated and (B) BA-treated WT mice.

Figure S6. BA generates splenomegaly in injected mice. Spleen weights normalized to body weight of WT, *IL-6*-KO, and *Hamp*-KO mice analyzed (A) 3 days and (B) 1 week after BA injection. Each bar represents the average weight of three mice. Error bars represent SD. *P* values were calculated using unpaired, 2-tailed Student's *t* test. **P*<0.05; ***P*<0.01.

Figure S7. Erythropoiesis in the spleen is spared by the inflammatory stimuli triggered by BA. (A) Schematic representation of the experiment: mice (WT) were bled (400 mL blood/25g mouse) for two consecutive days, then left to rest for two days, and on day 4 they were injected with $5x10^{8}$ particles of BA/mouse. On day 7 (3 days after BA administration), mice were sacrificed and their BM and spleen cells harvested for analysis of erythropoiesis by flow cytometry. (B) Hemoglobin values measured in mice at time 0, before injecting BA (day 4), and

at time of sacrifice (day 7) (N=4/PBS and N=5/BA). (C) FACS analysis of BM and spleen of mice 3 days after BA injection, using CD71, CD44 and Ter119 antibodies. Three mice per each group were analyzed by flow cytometry. One representative mouse per each group is shown. (D) The total number of cells in each erythroid population (I, II, III, IV, and V), derived from the percentages shown in C. Error bars represent SD. *P* values were calculated using unpaired, 2-tailed Student's *t* test. **P*<0.05; ****P*<0.001.

Figure S8. Destruction of RBC by macrophages. Example image of a macrophage (F4/80$^+$, green) that has internalized RBCs (Ter119$^+$, red), obtained by using the ImageStream X Mark II (Amins). Images were collected from the spleen of WT mice injected with either BA or PBS.

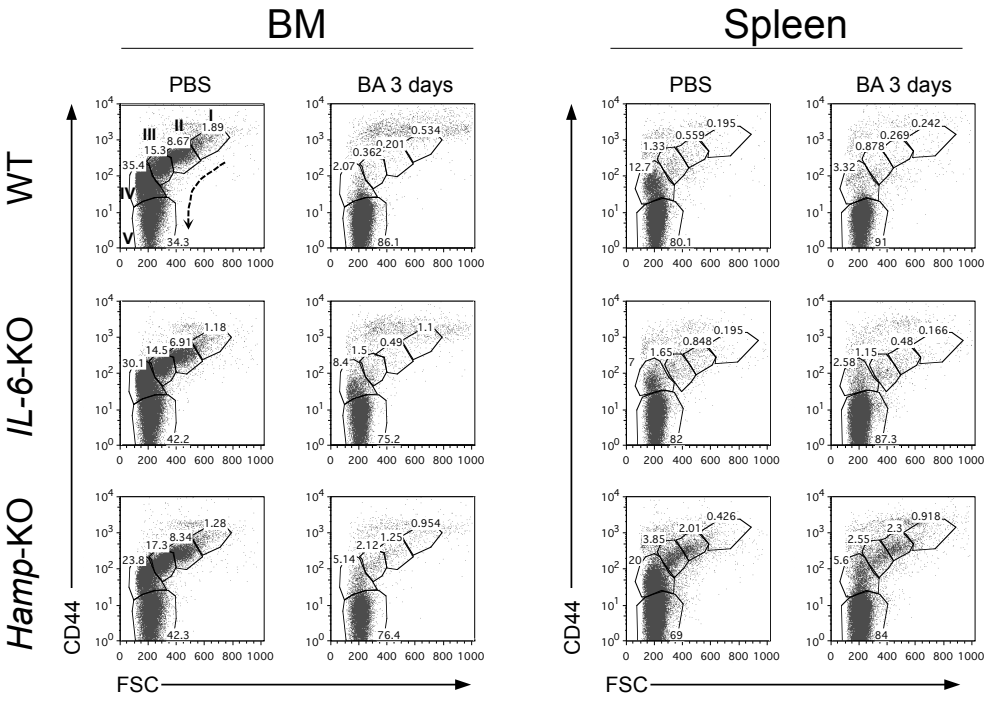

BM
Spleen
PBS
BA 3 days
PBS
BA 3 days
WT
IL-6-KO
Hamp-KO
CD44
FSC
I
II
III
IV
V
1.89
8.67
15.3
35.4
34.3
0.534
0.201
0.362
2.07
86.1
0.195
0.559
1.33
12.7
80.1
0.242
0.269
0.878
3.32
91
1.18
6.91
14.5
30.1
42.2
1.1
0.49
1.5
8.4
75.2
0.195
0.848
1.65
7
82
0.166
0.48
1.15
2.58
87.3
1.28
8.34
17.3
23.8
42.3
0.954
1.25
2.12
5.14
76.4
0.426
2.01
3.85
20
69
0.918
2.3
2.55
5.6
84

Figure S1

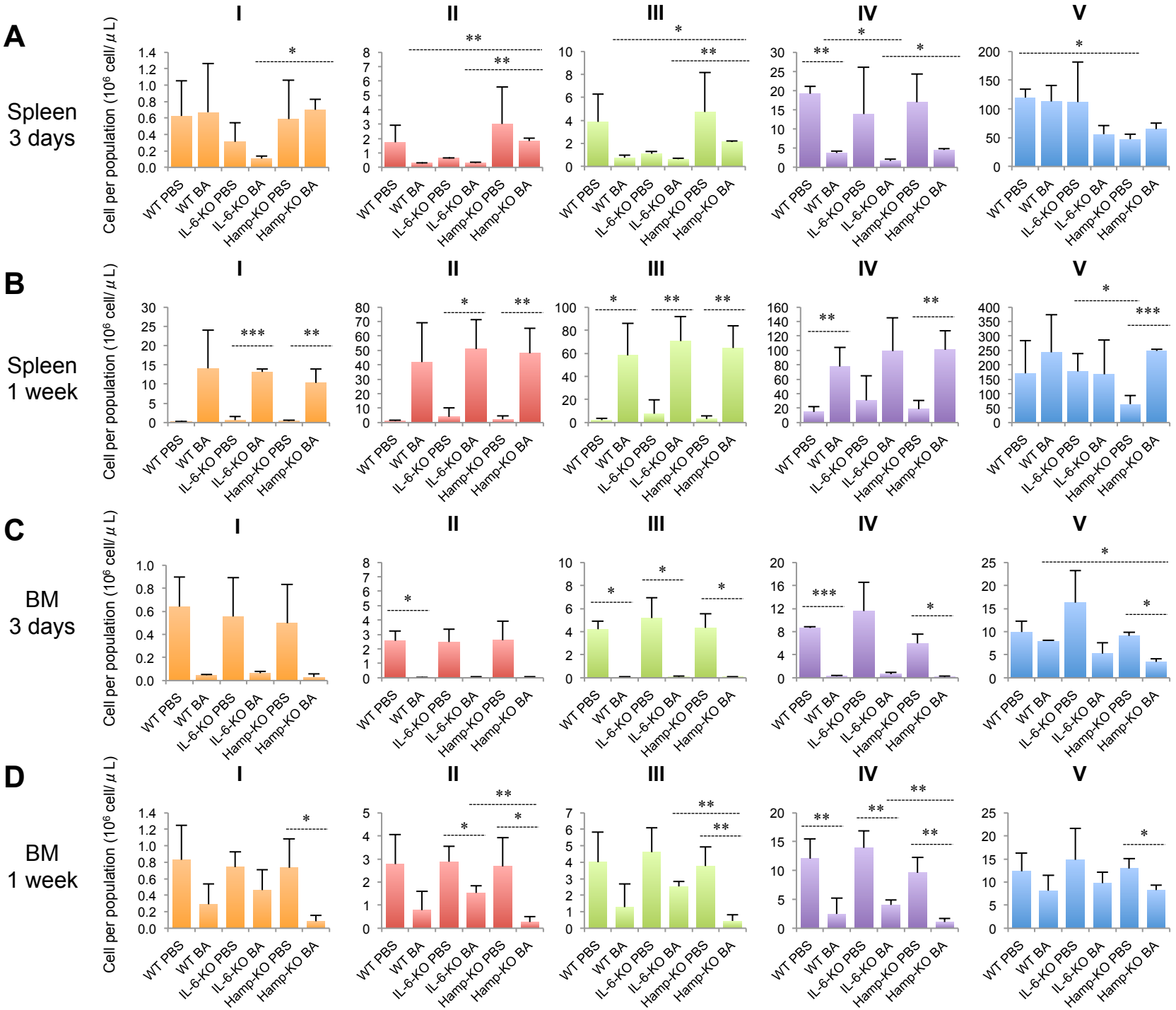

A
Spleen
3 days
B
Spleen
1 week
C
BM
3 days
D
BM
1 week
Cell per population (10⁶ cell/μL)
I
II
III
IV
V
WT PBS
WT BA
IL-6-KO PBS
IL-6-KO BA
Hamp-KO PBS
Hamp-KO BA

Figure S2

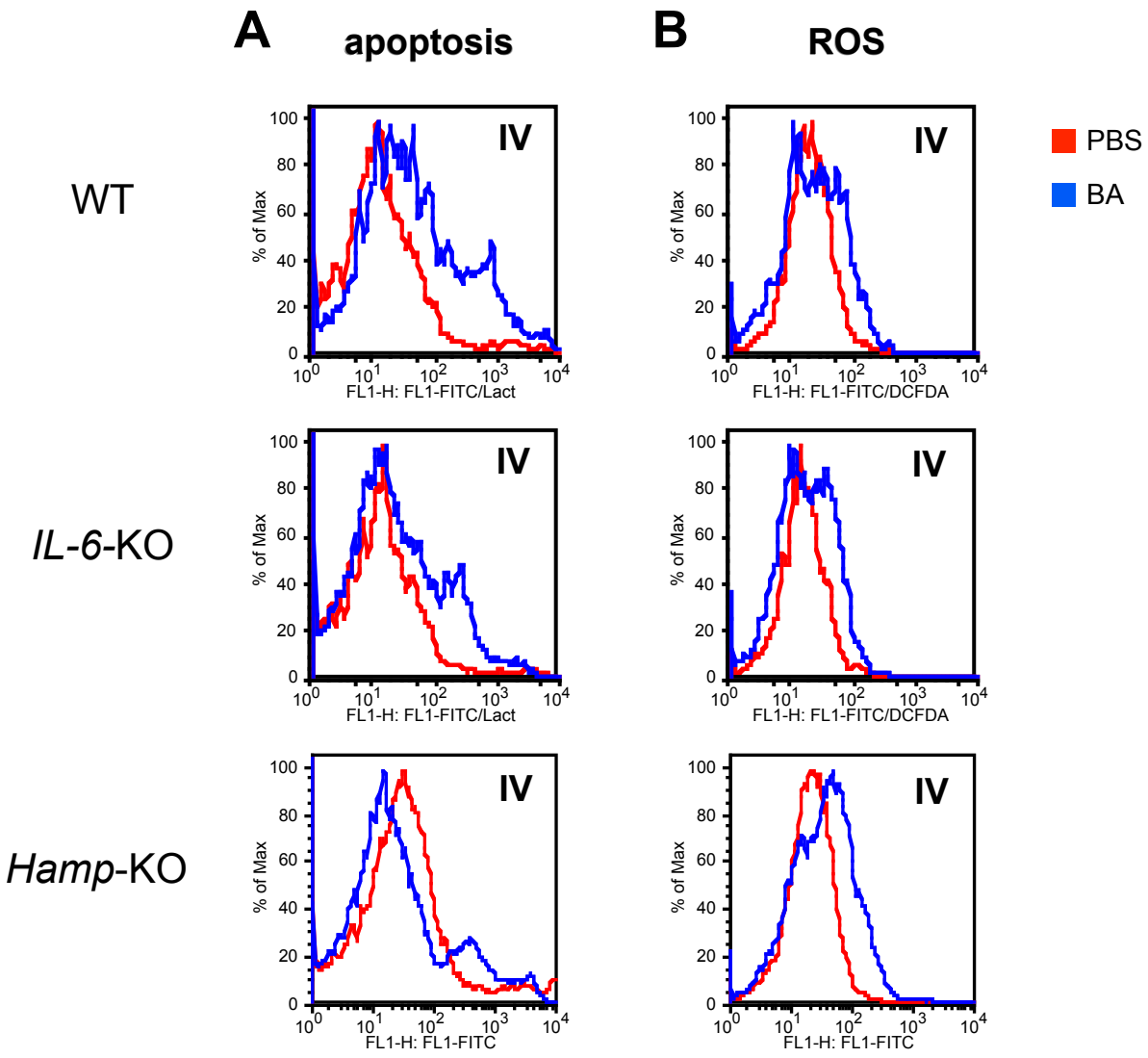
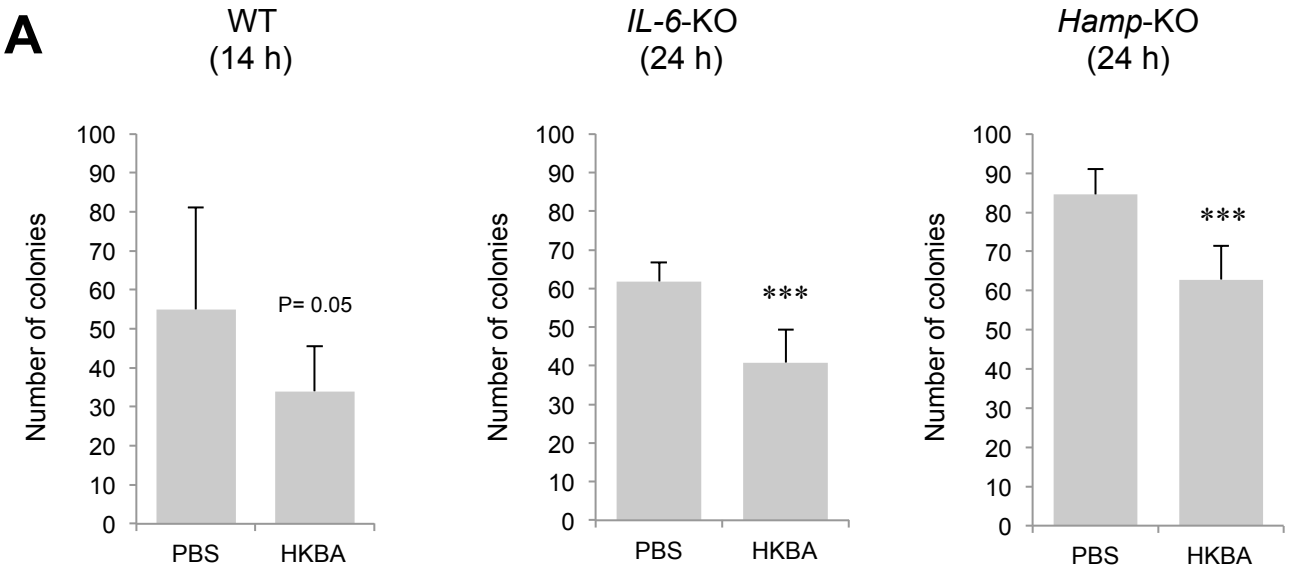

A
apoptosis
B
ROS
PBS
BA
WT
IL-6-KO
Hamp-KO
IV
% of Max
FL1-H: FL1-FITC/Lact
FL1-H: FL1-FITC/DCFDA
FL1-H: FL1-FITC

Figure S3

BFU-E



CFU-E

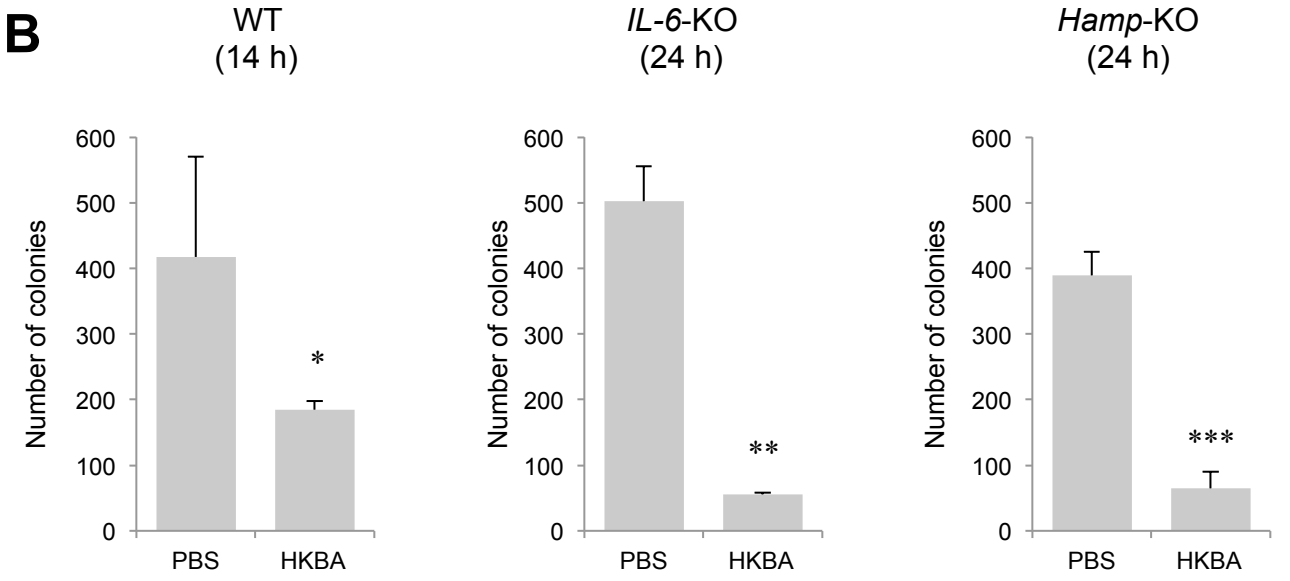


Figure S4

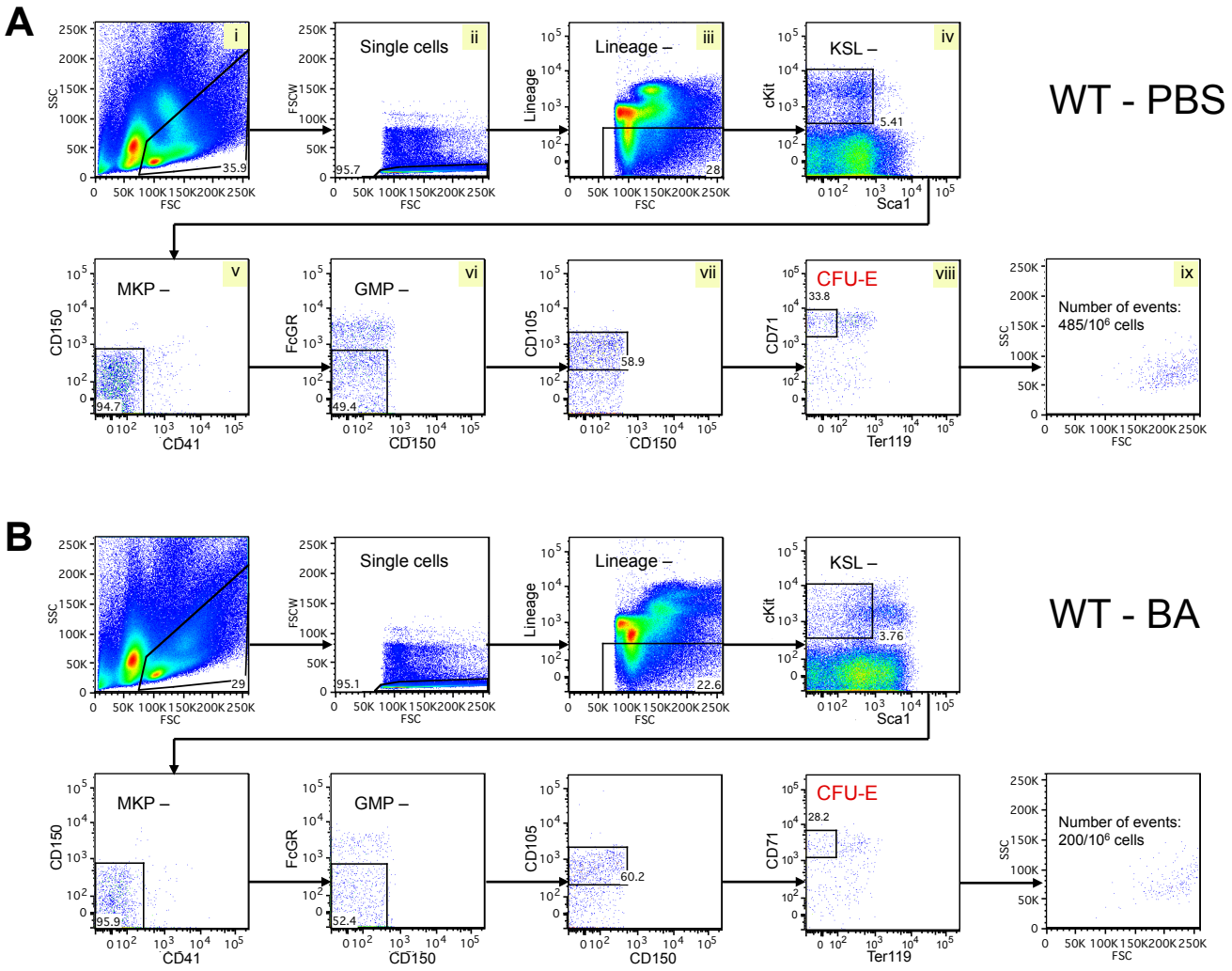

A
WT - PBS
i
SSC
FSC
35.9
Single cells
ii
FSCW
95.7
Lineage –
iii
Lineage
28
KSL –
iv
cKit
5.41
Sca1
MKP –
v
CD150
CD41
94.7
GMP –
vi
FcGR
CD150
49.4
vii
CD105
58.9
CD150
CFU-E
viii
CD71
33.8
Ter119
ix
Number of events:
485/10⁶ cells
B
WT - BA
29
95.1
22.6
3.76
95.9
52.4
60.2
28.2
200/10⁶ cells

Figure S5

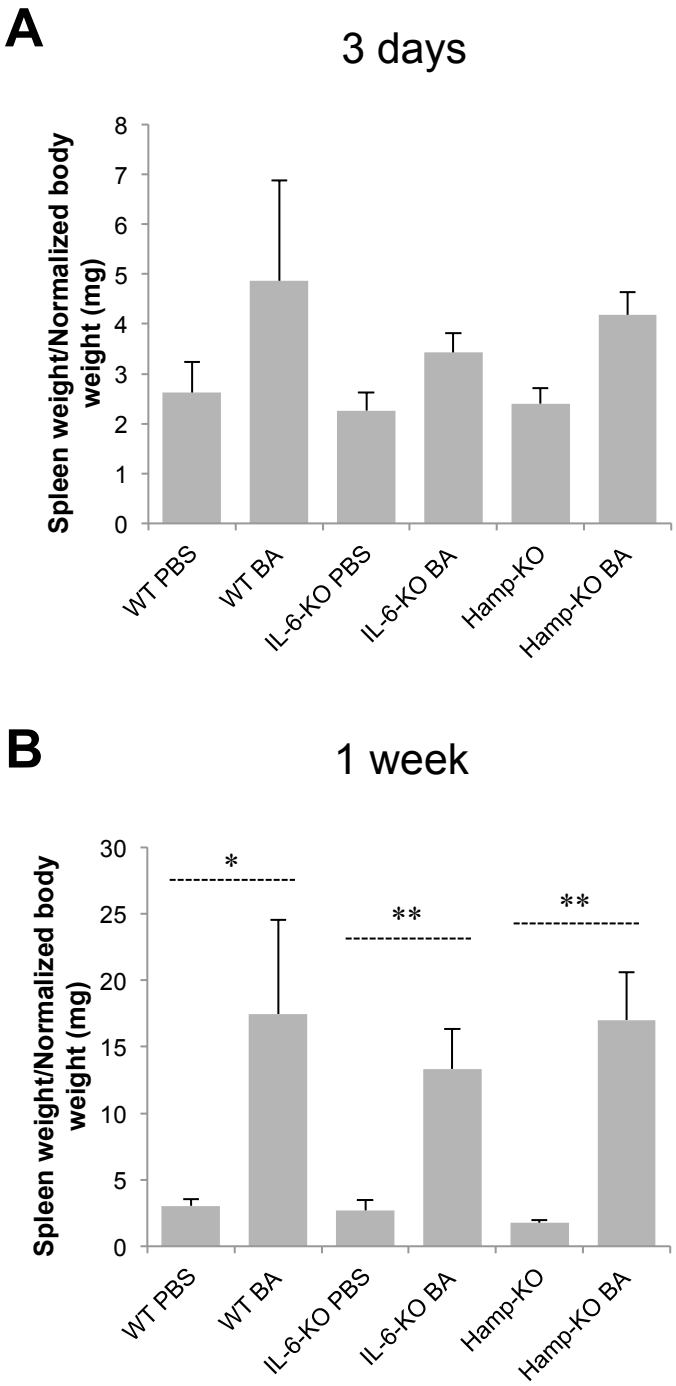

A
3 days
Spleen weight/Normalized body weight (mg)
0
1
2
3
4
5
6
7
8
WT PBS
WT BA
IL-6-KO PBS
IL-6-KO BA
Hamp-KO
Hamp-KO BA
B
1 week
Spleen weight/Normalized body weight (mg)
0
5
10
15
20
25
30
*
**
**
WT PBS
WT BA
IL-6-KO PBS
IL-6-KO BA
Hamp-KO
Hamp-KO BA

Figure S6

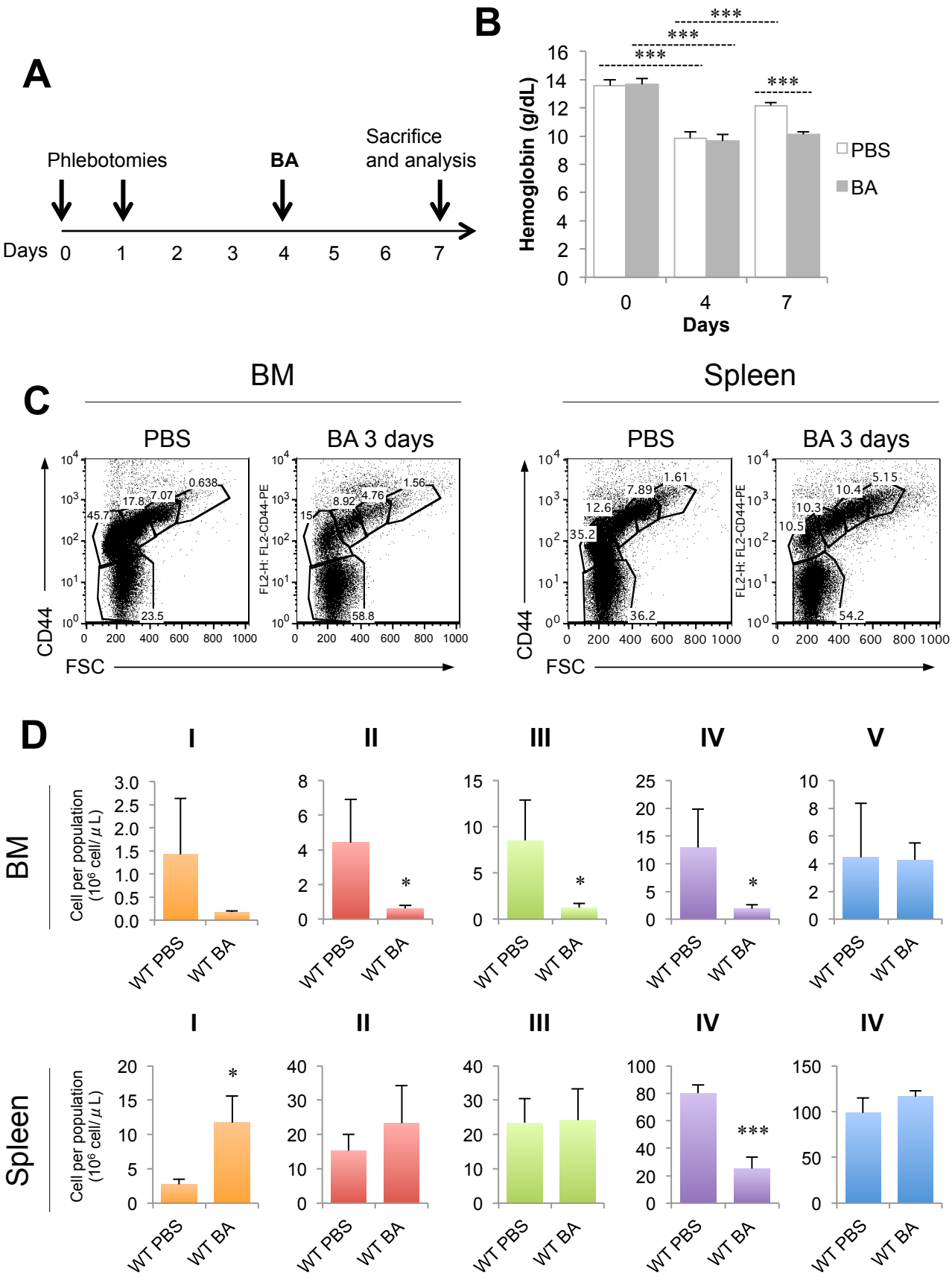

A
Phlebotomies
BA
Sacrifice and analysis
Days 0 1 2 3 4 5 6 7
B
Hemoglobin (g/dL)
Days
PBS
BA
***
C
BM
Spleen
PBS
BA 3 days
CD44
FSC
FL2-H: FL2-CD44-PE
D
I
II
III
IV
V
BM
Spleen
Cell per population (10^6 cell/ μL)
WT PBS
WT BA

Figure S7

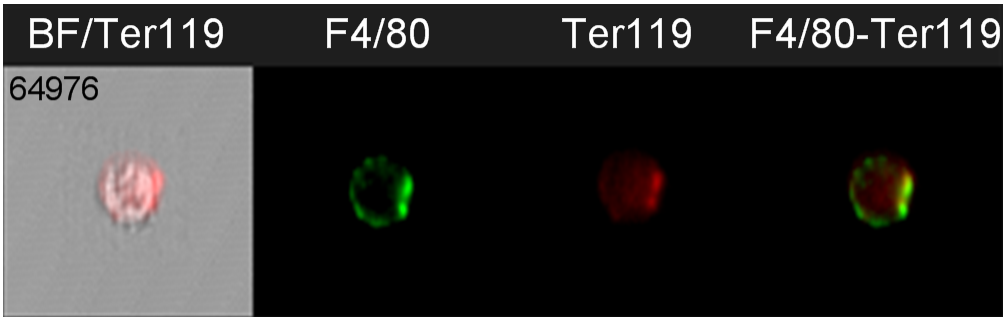


Figure S8